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Purification and Chemical Properties of *Acinetobacter calcoaceticus* A2 Biodispersan

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The extracellular dispersant of *Acinetobacter calcoaceticus* A2, referred to as biodispersan, was concentrated by ammonium sulfate precipitation and deproteinized by hot phenol treatment. The active component was an anionic polysaccharide (PS-A2). The specific activity of PS-A2 was approximately three times greater than that of crude biodispersan. PS-A2 had a sedimentation constant of 1.39 S, a diffusion coefficient of 18.8×10^{-8} cm^2 s^{-1}, and a partial molar volume of 0.65 cm^3 g^{-1}, yielding an average molecular weight of 51,400. Titration of the polymer gave two inflection points: $pK_1 = 3.1$ (1.15 μEq/mg) and $pK_2 = 8.0$ (0.4 μEq/mg). PS-A2 slowly consumed 1.10 μmol of periodate per mg. The ^{13}C nuclear magnetic resonance spectrum of PS-A2 indicated four methyl groups, four carbonyl C atoms, and four signals in the anomeric region (95 to 110 ppm), indicative of the presence of four different monosaccharides. Strong acid hydrolysis of PS-A2 yielded four reducing sugars: glucosamine, a 6-methyl aminohexose, galactosamine uronic acid, and an unidentified amino sugar. Ruthenium red binding to PS-A2 was stoichiometric: 1 molecule of dye bound per 2.0 carboxyl groups.

After growth of *Acinetobacter calcoaceticus* A2 on ethanol-salts medium, a nondialyzable dispersing agent, referred to as biodispersan, accumulates in the extracellular culture fluid (10). Since biodispersan, prepared by ammonium sulfate precipitation, was a crude mixture of polysaccharides and proteins, it became necessary to further purify the component(s) responsible for the biodispersing activity. Purification and chemical characterization of the active component are prerequisites for (i) studying structure-function relationships, (ii) developing chemical and immunological assays, (iii) developing a rational approach for optimizing fermentation conditions and strain improvement, and (iv) finding commercial applications for biodispersan.

In the studies presented here, the active component of biodispersan was purified and shown to be an anionic polysaccharide (PS-A2) with an average molecular weight of 51,400.

MATERIALS AND METHODS

Determination of dispersing activity. Dispersing activity was measured as described previously (10) except that 100 mg of milled Arad limestone was used in a final volume of 2.0 ml. After the 5% limestone suspension was allowed to equilibrate with the dispersing agent for 30 min, the mixture was vortexed for 30 s and then allowed to stand undisturbed for 30 min. The upper 1 ml was then removed for determination of turbidity in a Klett-Summerson colorimeter fitted with a green filter. Dilutions were made with distilled water so that the final Klett unit (K.U.) value was less than 150.

General analytical methods. Protein concentrations were determined by the method of Lowry et al. (7) with bovine serum albumin as a standard. Hexoses were determined by the indole-sulfuric acid method (2) with D-glucose as the standard. Hexosamines and reducing sugars were estimated after hydrolysis in 3 N HCl for 3 h at 100°C; total hexosamines were determined by the Partridge modification (18) of the Elson-Morgan method and by the nitrous acid-indole method (2), with galactosamine as the standard; and total reducing sugars were estimated by the Somogyi-Nelson arsenomolybdate method (14), with galactosamine as the standard. Periodate oxidation was followed spectrophotometrically (8) in a 2.0-ml solution (0.1 M acetate buffer, pH 4.5).

A Beckman model E analytical ultracentrifuge equipped with a Schlieren optical system was used for measurement of sedimentation velocity and diffusion constant at 19 ± 0.2°C. Absorbance was read on a Gilford model 240 spectrophotometer. Density measurements were performed with a digital densimeter (model DMA002C) (1).

Titrations were carried out with a pH meter by a microprocedure in which 0.01-ml portions of 0.1 N HCl or NaOH solutions were added with thorough mixing and exclusion of carbon dioxide to 4.0-ml solutions to be titrated.

Thin-layer chromatography was performed on coated cellulose plates (Merck) with solvent A (ethyl acetate-pyridine-water-acetic acid, 5:5:3:1, vol/vol/vol/vol) and solvent B (ethyl acetate-pyridine-water, 4:2:4, vol/vol/vol, upper phase) as developing solvents. Multiple chromatograms were searched for (i) sugars and polyols by alkaline silver nitrate (3) and (ii) amino sugars and amino acids by spraying with a 0.2% solution of ninhydrin in acetone, followed by heating at 105°C for 2 to 3 min.

Nuclear magnetic resonance (NMR) spectroscopy was performed with a Bruker AM-360 spectrometer with an Aspect 3000 computer and operating at 360.1 MHz and 90.5 MHz for ^1H and ^{13}C, respectively. Tetramethylsilane was used as a reference.

Materials. Preparation of the extracellular dispersing agent of *A. calcoaceticus* A2, referred to as biodispersan, was described previously (10). Preparation of PS-A2 from biodispersan is described in Results. Bovine serum albumin, DNase I, pancreatic RNase, and protease were products of Worthington Biochemicals Corp. Ruthenium red ($Cl_6H_{42}N_{14}O_2Ru_3$) was purchased from Merck, Darmstadt, Federal Republic of Germany). Bio-gel P-100, 50-100 mesh, Dowex 50 W (H^+ form) and Dowex 1 (OH^- form) were purchased from Bio-Rad Laboratories, Richmond, Calif. Other commercially available chemicals were reagent grade.

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RESULTS

Preparation and dispersing activity of polysaccharide PS-A2. Various samples of crude biodispersan contained 60 to 80% protein and 15 to 30% polysaccharide (by estimation of reducing sugar after strong acid hydrolysis). To ascertain whether the polysaccharide, protein, or both were required for dispersing activity, the polysaccharide was separated from the protein moiety by a modification of the hot phenol method (17). Crude biodispersan (10 g) dissolved in 500 ml of water was added to an equal volume of 90% phenol and then heated to 65 to 68°C. The mixture was stirred vigorously for 15 min at 65°C and then cooled to 20°C. The resulting emulsion was centrifuged at 4,000 × *g* for 30 min. The aqueous phase was transferred to a flask, and the remaining phenol layer and interface were extracted two more times with 500 ml of water. The combined water extracts were dialyzed extensively against several changes of distilled water and then freeze-dried to obtain a white fluffy material, referred to as PS-A2 (yield, 2.8 g).

Polysaccharide PS-A2, prepared as described above, contained less than 5% protein. A comparison of the dispersing activity of PS-A2 and crude biodispersan is shown in Fig. 1. The specific activity of PS-A2 was approximately three times greater than that of the crude biodispersan. Since the yield of PS-A2 from biodispersan was 28%, it follows that the recovery of dispersing activity was approximately 84%. The activities of biodispersan and PS-A2 were not reduced by treatment with proteases or nucleases. Additional treatment of PS-A2 with hot phenol reduced the protein content to 0.1% without lowering its specific dispersing activity. Thus, the dispersing activity of biodispersan was due to polysaccharide PS-A2.

The effect of pH and salts on the dispersing activity of PS-A2 was similar to that described (10) for crude biodispersan. The dispersing activity of PS-A2 was stable to high temperature (up to 120°C for 15 min at pH 7.0) and alkali conditions (1 N NaOH, 100°C, 30 min). In 1 N HCl at 100°C, the dispersing activity of PS-A2 was reduced by 70, 90, and 100% after 2, 10, and 30 min, respectively. In 0.1 N HCl at 100°C, the activity was reduced by 50% in 5 min.

Physical and chemical characterization of PS-A2. Polysaccharide PS-A2 was retained by an Amicon XM-30 filter, indicating a molecular weight of over 30,000. Dispersing activity eluted from a Bio-Gel P-100 column as a broad peak with an apparent molecular weight of 55,000.

Sedimentation velocity analysis of 2 mg of PS-A2 per ml in 0.02 M Tris buffer (pH 7.4) showed a single broad band corresponding to an $s_{20,w}$ of 1.39 S. The diffusion coefficient, D, also determined in the analytical centrifuge, was 18.8×10^{-8} cm^2 s^{-1}. The partial specific volume, V, of PS-A2 was 0.65 cm^3 g^{-1}. Estimating the molecular weight of PS-A2 from the equation $M = RTs/D(1 - VP)$, where R is the gas constant, T is the absolute temperature, and P is the density of the solution, yielded an average molecular weight of 51,400.

The UV absorption spectrum of 1 mg of PS-A2 per ml (205 to 350 nm) showed a single maximum at approximately 210 nm (A_{210}, 0.8). The absorbance was probably due to the *N*-acetyl group of amino sugars because *N*-acetyl galactosamine also had a maximum at 210 nm with a similar extinction coefficient.

Polysaccharide PS-A2 consumed 1.10 μmol of periodate per mg. Periodate uptake ceased after 5 h at 30°C, pH 4.5. The periodate-treated material did not lose any dispersing activity.

Titration of PS-A2 (12 mg/4 ml) between pH 2.5 and 11.0 showed inflection points, corresponding to pK_1 of 3.1 (identical to glucuronic acid) and pK_2 of 8.0 (similar to glucosamine). From the titration data, the quantity of pK_1 3.1 and pK_2 8.0 components was 1.15 μEq/mg and 0.4 μEq/mg of PS-A2, respectively.

Binding of ruthenium red to PS-A2. The binding of polycationic ruthenium red to polyanions has been used to help

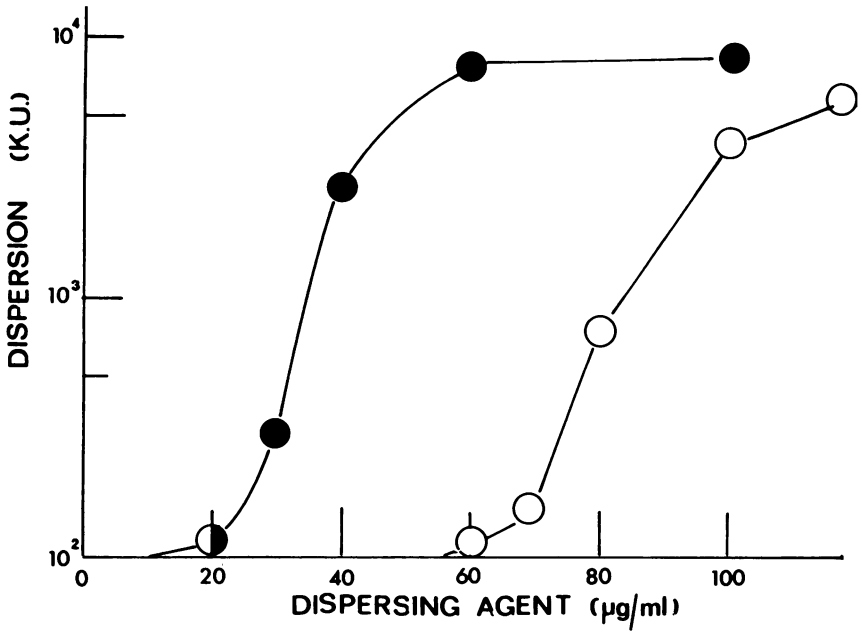

FIG. 1. Limestone dispersing activity of polysaccharide PS-A2. Dispersion (corrected Klett units [K.U.]) as a function of the concentration of crude biodispersan (○) and polysaccharide PS-A2 (●) was determined as described in Materials and Methods, with 200 mg of milled limestone in a total volume of 4 ml.

visualize DNA and pectin in the electron microscope (4). When a solution of ruthenium red was mixed with PS-A2 and then passed through a mixed-bed ion-exchange resin, a portion of the dye was adsorbed to the resin (free dye) and a portion was not adsorbed (bound to PS-A2). As seen in Fig. 2, the amount of nonadsorbed dye was directly proportional to the concentration of PS-A2, indicating that ruthenium red binding can be used as an alternative assay for PS-A2. From the linear portion of the curve and molar extinction of ruthenium red, it can be calculated that 0.58 μmol of dye was bound per mg of PS-A2. Since PS-A2 contained 1.15 μEq of carboxyl groups per mg, each dye molecule was bound to an average of 2.0 carboxyl groups.

The binding of ruthenium red to crude biodispersan was complex. At low concentrations of biodispersan (0 to 0.1 mg/ml), relatively low amounts of dye were bound (0.073 μmol of dye per mg of biodispersan). With biodispersan between 0.1 and 0.5 mg/ml, the ratio of dye bound was constant, 0.19 μmol/mg. The latter value was close to the value predicted from the fraction of PS-A2 in crude biodispersan, suggesting that the contaminating protein does not contribute to dye binding.

NMR spectroscopy. The ^{13}C NMR spectrum of polysaccharide PS-A2 showed 23 peaks (Table 1). The signals at 24.8, 24.7, and 23.2 ppm indicate the presence of the methyl C atoms of three acetyl groups. Since the polysaccharide did not contain *O*-acetyl groups, these signals arose from the *N*-acetyl groups of amino sugars. The signal at 18.2 ppm is typical of the methyl C atom of a 6-deoxyhexose such as *N*-acetylfucosamine (11). Three of the carbonyl C atoms arose from the corresponding *N*-acetyl groups; the fourth carbonyl C atom must be due to the free carboxyl group of the uronic acid (from titration data). The anomeric region between 95 and 110 ppm contained four signals, indicative of the presence of four different monosaccharide units.

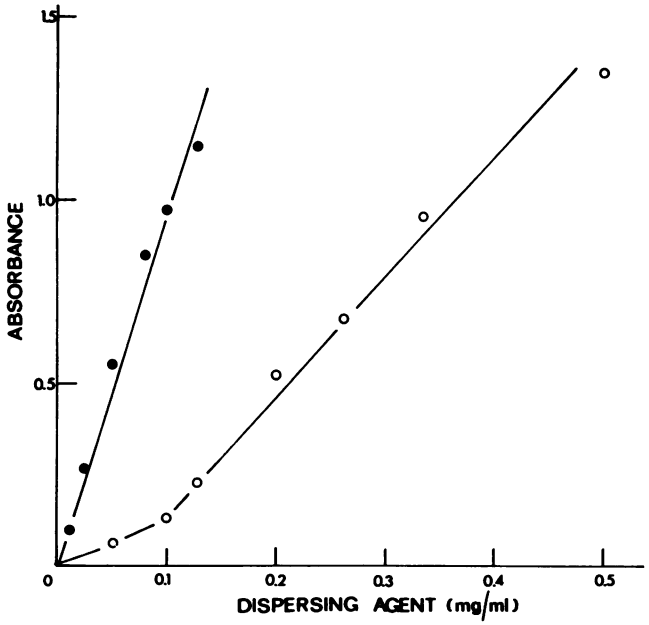

FIG. 2. Binding of ruthenium red to PS-A2. To conical tubes containing 3 ml of ruthenium red (0.1 mg/ml) was added 3 ml of solutions of either PS-A2 (●) or biodispersan (○). The solution was then mixed with a mixture of Dowex 50 and Dowex 1. After the resin settled, the clear supernatant fluid was removed for determination of absorbance at 533 and 600 nm. The difference spectrum ($A_{533} - A_{600}$) is plotted as a function of dispersant concentration.

TABLE 1. Signal assignments in ^{13}C NMR spectrum of polysaccharide PS-A2

Carbon assignment	Signal(s) (ppm)
Carbonyl	177.9, 177.4, 176.6, 176.1
Unknown	143.0
Anomeric C-1	102.9, 102.4, 100.0, 99.4
C-OH	81.5, 80.6, 79.5, 73.6, 72.7, 69.7, 62.3, 56.2, 54.2, 50.3
Methyl	24.8, 24.7, 23.2, 18.2

Acid hydrolysis of PS-A2. Preliminary hydrolysis studies were performed on PS-A2 with concentrations of hydrochloric acid varying from 0.1 to 6.0 M. After removal of HCl in vacuo, the products were examined for reducing power and sugars and amino sugars by thin-layer chromatography. A maximum of 42% reducing sugar (with glucose as the standard) was obtained after 3 h in 3 M HCl at 100°C. Even under these conditions, large amounts of PS-A2 were incompletely hydrolyzed. This was demonstrated by eluting material from the origin of the chromatograph and hydrolyzing it a second time. Additional amino sugar monosaccharides were released from the incompletely hydrolyzed oligosaccharide fraction. Hydrolysis for longer periods of time or with stronger acid resulted in further destruction of the sugars.

Estimation of the quantity of amino sugar in acid-hydrolyzed PS-A2 by the nitrous acid-indole and Elson-Morgan methods gave 14 and 36%, respectively. In the nitrous acid-indole test, the spectrum of the color product was shifted from 492 nm (for glucosamine) to 460 nm for the mixture of amino sugars present in PS-A2.

Chromatography of sugar components of PS-A2. Table 2 summarizes the thin-layer chromatographic behavior of the acid hydrolysis products of PS-A2. Each of the four silver nitrate-positive components was also ninhydrin positive, indicating the presence of four different aminosugars. Component i has been tentatively identified as glucosamine by its behavior on standard ion-exchange amino acid analysis columns (16), in addition to its movement by thin-layer chromatography in solvents A and B. Component ii moved more rapidly than any of the hexosamines, suggesting that it is a pentosamine or a 6-methyl aminohexose, such as rhamnosamine or fucosamine. Component iv had the same R_G

TABLE 2. Properties of sugar products of PS-A2 hydrolysis[a]

Component	R_G[b]	Ninhydrin reaction[c]
Standards		
Glucosamine	1.00	Purple
Galactosamine	0.87	Purple
Glucose	1.50	—
Galactose	1.33	—
Glucoronic acid	0.58	—
Acid hydrolysis products of PS-A2		
i	0.99	Purple
ii	1.38	Purple
iii	0.69	Blue
iv	0.27	Tan

[a] Obtained after 3 h of hydrolysis in 3 N HCl at 100°C.
[b] Rate of movement of each sugar relative to glucosamine in solvent A.
[c] All components had positive alkaline silver nitrate tests. Spot tests were determined directly on the chromatograms. —, No ninhydrin reaction.

and ninhydrin staining reaction as galactosamine uronic acid (19) in solvents A and B. The identity of component iii, which gave a blue color with ninhydrin and a weak silver nitrate test, is unknown.

DISCUSSION

The extracellular dispersing factor of *A. calcoaceticus* A2 was concentrated by ammonium sulfate precipitation and deproteinized by hot phenol treatment. The resulting acidic polysaccharide preparation (PS-A2) was free of protein and nucleic acids and contained 84% of the initial dispersing activity. From sedimentation and diffusion data, the average molecular weight of PS-A2 was 51,400. This was consistent with the observation that the dispersing activity eluted from gel permeation columns as a broad (heterogeneous) peak with an approximate molecular weight of 55,000.

^{13}C NMR and thin-layer chromatography analysis of acid-hydrolyzed PS-A2 indicated that the polysaccharide contained four different amino sugars. Titration curves and ^{13}C NMR analysis showed that the amino sugars were *N*-acetylated. Only 8.7% (0.4 μEq/mg) of the amino groups in the polymer were titratable. One of the amino sugars had the properties of galactosamine uronic acid (1.15 μEq of carboxylate ion per mg of PS-A2). If a molecular weight for *N*-acetyl galactosamine uronic acid of 222 is assumed, it would constitute 25.5% of the polymer. Direct estimation of the other amino sugars in PS-A2 is not possible at this time because the hydrolysis conditions necessary to release them from the polymer cause considerable decomposition of the amino sugars. Resistance to hydrolysis of polysaccharides containing amino sugars and uronic acids is well known (12).

Rough estimates (from intensities of reducing and ninhydrin-positive materials on chromatograms and ^{13}C NMR anomeric carbon signals) indicate that the four amino sugars were present in equal amounts. One of the four sugars contained vicinyl hydroxyl groups, as determined by periodate uptake.

A spectrophotometric assay was developed for measuring ruthenium red binding to PS-A2. The assay was based on the observation that the polysaccharide-bound dye was not adsorbed by ion-exchange resins. The stoichiometry of the reaction was 1 molecule of dye per 2 carboxyl groups in the polymer. Although the precise mechanism by which ruthenium red binds polyanions has not been established, it is clear that not only the content of the carboxyl groups but also the distance between them affects the reaction (4, 15).

It is interesting to compare the chemical structure and activity of PS-A2 with the previously characterized exopolysaccharides of *A. calcoaceticus* RAG-1 (19) and BD4 (5). All three heteropolysaccharides are anionic due to uronic acid residues. PS-A2, which disperses limestone but does not emulsify hydrocarbons, has a relatively low molecular weight of 51,400. Emulsan from RAG-1 and PS-4 from BD4 are potent emulsifying agents (6, 9) and have molecular weights of ca. 1,000,000 (6, 19). The high molecular weights of emulsan and PS-4 are required for their activities, since specific enzymes which introduce a few breaks into these molecules caused a complete loss of emulsifying activity (6, 13). Apparently, for an *A. calcoaceticus* surface-active agent to stabilize hydrocarbon-in-water emulsions, the polymer must be amphipathic and have a high molecular weight, whereas to function as a dispersant of inorganic solids, the polymer can have a lower molecular weight but must contain a high charge density.

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